The Chemical Synthesis of Amino Acyl Adenylates*

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The present paper deals with a description of the method of preparation and partial purification of several amino acid adenylates. Two procedures for the synthesis of these compounds have previously been described, but these have certain disadvantages for general application. The first of these methods (1) which leads to the synthesis of the leucyl, alanyl, and phenylalanyl adenylates in yields of about 10 per cent, involved the reaction of the amino acid acyl chloride with the silver salt of adenosine 5′-phosphate (A5P).[1] In another method, Wieland *et al.* (2) used DL-valine thiophenol hydrochloride as the activated amino acid and they were able to effect a transfer of the valine moiety to A5P in yields of 10 to 20 per cent.

Recently the usefulness of N,N′-dicyclohexylcarbodiimide for the synthesis of nucleoside pyrophosphate derivatives was elegantly demonstrated by Khorana *et al.* (3–5). Earlier, Zetzche and Fredrich (6) had used the carbodiimides for the synthesis of carboxylic acid anhydrides. It seemed, therefore, that the carbodiimides might offer a useful reagent for coupling the amino acids to A5P by an acyl phosphate linkage. Soon after our studies were under way (7) Talbert and Huennekens (8) reported the synthesis of butyryl adenylate with DCC. The method to be described here involves the use of DCC to effect a condensation of the carboxyl group of a free amino acid with the phosphate of adenylic acid in aqueous pyridine. Using this procedure the adenylate derivatives of D- and L-methionine, L-phenylalanine, L-tryptophan, and L-serine have been prepared and purified.

MATERIALS AND METHODS

Crystalline A5P (free acid) was obtained from the Sigma Chemical Co. and the L-amino acids were products of the California Foundation for Biochemical Research or of Nutritional Biochemical Corp.

A5P deaminase was prepared by the procedure for Preparation A of Kalckar (9) and dialyzed against 0.05 M potassium succinate buffer, pH 6.0, to remove ammonium sulfate.

Hydroxylamine was freshly prepared by neutralizing a stock solution of 4 M hydroxylamine hydrochloride with 3.5 N NaOH to a pH of 6.5.

The concentration of the amino acid adenylates was measured spectrophotometrically by conversion to the amino acid hydroxamates. To 0.5 ml. of neutralized hydroxylamine (2 M) were added the amino acyl adenylate and water to a total volume of 1 ml. After three minutes 1 ml. of a solution of acidic ferric chloride (10) was added and the mixture was shaken rapidly to remove gas bubbles, filtered, and the optical density at 540 mμ was measured against a blank containing no amino acid adenylate. The concentration was calculated with extinction coefficients obtained with synthetic amino acid hydroxamates.

Total A5P was determined with A5P deaminase (9) after preliminary hydrolysis of an aliquot of the amino acid adenylate at pH 10 for 5 minutes at room temperature. Free A5P (in the presence of amino acyl adenylate) was determined with a large amount of A5P deaminase to complete the reaction in 1 to 2 minutes, and thus minimize the slow liberation of A5P due to destruction of the amino acid adenylate.

Ribose was determined by the Mejbaum method (11) with A5P as the standard. Methionine was determined by a modification of the method of McCarthy and Sullivan (12), and phosphate was measured by the method of Fiske and SubbaRow (13).

RESULTS

DCC in aqueous pyridine brings about the formation of the substituted acyl phosphate derivative from an amino acid and A5P. With methionine, for example, the reaction proceeded to completion (Table I). The final value attained depended upon the amount of methionine or A5P employed and remained constant for at least 90 minutes. Whether this is due to the stability of the methionyl adenylate under these conditions or to the attainment of a steady state in which the rate of breakdown was equal to the rate of synthesis is not known. A detailed description of the preparation and isolation of L-methionyl adenylate follows.

Synthesis of L-methionyl Adenylate—L-Methionine (2.0 mmoles) and A5P (1.92 mmoles) were mixed with 3.2 ml. of cold water and 10.4 ml. of pyridine in a 250 ml. glass-stoppered flask. 8 N HCl (0.25 ml.) was added and the mixture was stirred in an ice bath with the aid of a magnetic stirrer. DCC (50 mmoles), dissolved in 12 ml. of pyridine, was added and the mixture was stirred vigorously. The formation of L-methionyl adenylate was determined on aliquots removed at various time intervals (see "Methods"). After 3 to 3.5 hours there was no further increase in methionyl adenylate formation. The value attained was usually between 90 and 95 per cent of the theoretical maximum based on the amount of A5P used.

The reaction was terminated and the crude methionyl adenylate was precipiated by the addition of about 150 ml. of acetone chilled to −15°. After 45 seconds the precipitate was filtered rapidly with the aid of suction, washed with small portions of a mixture of acetone-alcohol (60:40) at 0°, then with ether (0°), and sucked almost dry on the filter. The material was then dried further at 3° overnight *in vacuo* over P_2O_5 and paraffin. The precipitation, washing, and air drying were completed in approximately 8 minutes. The material obtained at this stage

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[1] The abbreviations used are: A5P, adenosine 5′-phosphate; ATP, adenosine triphosphate; DCC, dicyclohexylcarbodiimide.

weighed 1.77 gm. and contained N,N′-dicyclohexylurea, L-methionine, A5P, a trace of pyridine hydrochloride, and methionyl adenylate in about 40 to 50 per cent yield based on the A5P consumed. Even with the precautions of working at low temperatures and rapid filtration, there seems to have been appreciable breakdown of the amino acid adenylate.

The crude material was evenly suspended in cold water at a concentration of 100 mg. per ml. and filtered to remove the insoluble dicyclohexyl urea. The residue was washed with 5 ml. of cold water and the wash and original filtrate were combined. The pH was adjusted to 3 to 4 with HCl and 85 ml. of cold ethanol (−15°) were added. After 1 hour at −15°, the precipitate was filtered, washed with cold ethanol, and dried *in vacuo* at 3°. The yield was 670 mg. and this contained 0.6 mmole of methionyl adenylate with a purity of 46 per cent based on the optical density at 260 mμ. At this stage the methionyl adenylate was stable for periods of at least 2 months when kept dry and cold.

The removal of A5P and further purification of the methionyl adenylate was carried out as follows. About 100 to 200 mg. were dissolved in 2 to 4 ml. of cold water and the pH was adjusted to 4 to 4.5 with solid potassium bicarbonate. The solution was passed through a Dowex 1 column (Cl^- form, 10 per cent cross-linked, 1 × 4 cm.) and the column was then washed with 1 to 2 ml. of water. The wash and original liquid which had passed through the column were combined, adjusted to pH 4.5, and used as such. Under the above conditions, free A5P was quantitatively adsorbed to the column, whereas 60 to 80 per cent of the methionyl adenylate appeared in the effluent (1). The methionyl adenylate concentration decreased about 5 per cent per day when the material was kept frozen.

Properties and Analysis of Methionyl Adenylate—Spectrophotometric examination of this solution revealed an ultraviolet absorption spectrum which was essentially indistinguishable from that of free A5P. The ratios of the absorption at 280 to 260 mμ and 250 to 260 mμ at pH 2 were 0.22 and 0.84, respectively,

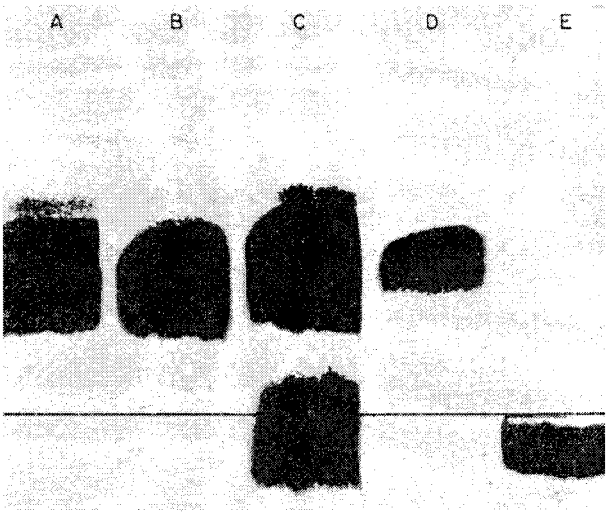

FIG. 1. Paper electrophoretic separation of methionyl adenylate and A5P: The separation was carried out on Whatman 3MM paper using 0.02 M sodium citrate buffer, pH 3.1, and a voltage of 14 volts per cm. for 3 hours at 3°. *A*, crude L-methionyl adenylate exposed to 0.01 N KOH for 5 minutes at 25°; *B*, crude L-methionyl adenylate treated with 2 M hydroxylamine; *C*, crude L-methionyl adenylate; *D*, A5P marker; and *E*, purified L-methionyl adenylate. The spots were visualized and the photographs taken with an ultraviolet lamp (2540 A). The small band sometimes found in crude methionyl adenylate preparations has not been identified but may be diadenosine 5′-pyrophosphate (15).

TABLE I

Synthesis of L-methionyl adenylate with DCC

A5P	L-Methionine	L-Methionine adenylate formed*
mmoles	*mmoles*	*mmoles*
1.0	5.0	1.01
0.0	5.0	0.0
1.0	1.0	0.92
0.31	0.75	0.30
0.31	0.40	0.29
0.31	0.14	0.14

* Measured as methionine hydroxamate as described in "Methods."

TABLE II

Analysis of L-methionyl adenylate

Adenine*	Ribose	Total P	A5P		Methionine	
			Free	Bound	Bound†	Total
1.00	0.98	0.97	0.03	0.99	0.97	1.10

* The values are expressed as ratios based on adenine which was determined by the optical density at 260 mμ at pH 2 using an extinction coefficient of 15.1 × 10^3 $cm^{-1}M^{-1}$ (14).

† Determined as methionine hydroxamate as described in "Methods."

TABLE III

Synthesis of amino acyl adenylates

Amino acyl adenylate	Fraction	Yield	Spectral purity*	Spectral ratio	
				280 mμ/260 mμ	250 mμ/260 mμ
		%	%		
L-Seryl adenylate	Acetone precipitate	45	53		
	Alcohol precipitate	32	46		
	Unadsorbed by Dowex	25	94	0.22	0.85
L-Phenylalanyl adenylate	Acetone precipitate	52	56		
	Alcohol precipitate	30	45		
	Unadsorbed by Dowex	23	98	0.23	0.86
L-Tryptophanyl adenylate	Acetone precipitate	57	60		
	Alcohol precipitate	40	57		
	Unadsorbed by Dowex	28	97	0.39	0.86
D-Methionyl adenylate	Acetone precipitate				
	Alcohol precipitate				
	Unadsorbed by Dowex	29	95	0.21	0.84

The fractions are the same as those described for the synthesis of L-methionyl adenylate.

* The purity is based on the ratio of the amount of amino acyl adenylate to total A5P.

for methionyl adenylate, compared to 0.22 and 0.84 for free A5P (14).

Analyses for the various constituents of the purified methionyl adenylate (Table II) show reasonably good agreement between the A5P, total methionine, and bound methionine. Occasionally, certain preparations were contaminated with more free methionine than shown in Table II. This, however, rarely exceeded a value of 20 per cent free methionine.

Paper electrophoresis studies with methionyl adenylate showed it to be slightly cationic at pH 3.1 and easily separable from A5P which migrates as an anion under these conditions (Fig. 1). Exposure of methionyl adenylate to neutral hydroxylamine or 0.01 N KOH for 5 minutes at room temperature resulted in the disappearance of the methionyl adenylate and formation of A5P.

Synthesis of Other Amino Acyl Adenylates—The amino acyl adenylates of L-serine, L-phenylalanine, L-tryptophan, and D-methionine have been prepared with the use of the same procedure already described for methionyl adenylate. The data for recoveries, purity, and similar properties are summarized in Table III.

DISCUSSION

The alkyl carbodiimides have proved to be extremely usefu reagents for the synthesis of a number of compounds of biological interest. In addition to the nucleotide pyrophosphate derivatives (3-5), the unsymmetrical nucleotide pyrophosphate coenzymes, such as cytidine diphosphocholine (16), diphosphopyridine nucleotide (17), flavinadenine dinucleotide (18), and, through the nucleoside 5′-phosphoramidate, uridine diphosphoglucose (19), have been prepared with DCC. More recently this reagent has been utilized for the synthesis of a deoxydinucleoside monophosphate (20) and dideoxynucleotides (21).

In the present case DCC has been employed to link an amino acid to A5P. The studies with L-methionyl adenylate indicate that the linkage is an anhydride between the amino acid carboxyl group and the phosphate of A5P (Fig. 2). The evidence for this conclusion is based on the following properties. The purified compound contains A5P and methionine in a 1:1 ratio. The absorption spectrum is identical to that of free A5P, indicating that the amino acid is not linked to the adenine group. At pH 3.1 the compound moves slowly as a cation on paper electrophoresis and it is not retained by the strongly cationic adsorbent Dowex 1. The remaining uncertainty in the proof of structure is in the position of the amino acyl group. It could reside in an ester linkage on the 2′- or 3′-hydroxyl group of the ribose or as shown in Fig. 2 in an anhydride linkage with the 5′-phosphate group. De Moss *et al.* (1) have used as evidence for a linkage with the 5′-phosphate group the inability of adenylic deaminase

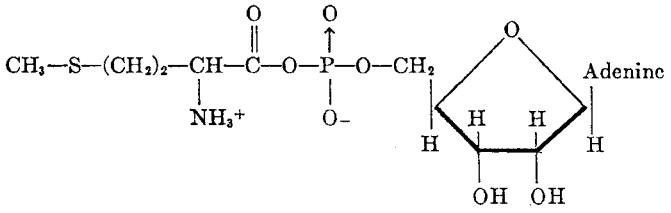

FIG. 2

to deaminate the amino acid adenylate derivative. However, it is not clear whether substitution in the 2′ or 3′ position on the ribose would likewise prevent the action of adenylic deaminase.

The rapid and quantitative formation of the amino acid hydroxamate in the presence of hydroxylamine at pH 6.5 would appear to argue in favor of the anhydride linkage. It has been pointed out recently (22), however, that amino acid esters also react with neutral hydroxylamine to form the hydroxamate. It should be emphasized however that Raacke (22) has pointed out that at pH 7 and below the rate of amino acid hydroxamate formation is very slow and usually incomplete. With the amino acyl adenylates the reaction is complete within a few minutes, a behavior which is more characteristic of the anhydride. Moreover the enzymatic formation of ATP from the amino acyl derivatives and inorganic pyrophosphate is more easily reconciled with the formulation shown in Fig. 2. Similar evidence for the structure of L-leucyl adenylate has been presented by De Moss *et al.* (1).

Although the detailed description for the preparation of only a few of the amino acid adenylates is presented here, preliminary experiments with leucine, valine, isoleucine, alanine, glycine, threonine, tyrosine, and arginine have demonstrated that these too are converted to the adenylate derivatives under conditions similar to those described above. With some of these amino acids the reaction proceeded more slowly and the final value reached was only 30 to 60 per cent of the theoretical maximum. Attempts to prepare the histidyl, glutamyl, and aspartyl derivatives of adenylic acid have been unsuccessful to date. The reasons for this are not clear but in the case of the dicarboxylic acids there is the possibility of internal cyclization to form 5 and 6 membered cyclic anhydrides which might be unstable in aqueous pyridine. With regard to histidine, it has been shown (23) that imidazole catalyzes a rapid breakdown of the acyl adenylate derivatives and it is conceivable that the imidazole group of histidine might promote the breakdown of a carboxyl activated histidine in the aqueous pyridine system. It does seem possible however, that with further work including the use of suitable protected derivatives of the amino acids, all of the naturally occurring amino acids could be converted to the adenyl derivatives with DCC.

SUMMARY

The present paper describes the chemical synthesis of the methionyl, seryl, phenylalanyl, and tryptophanyl adenylates from the free amino acids and adenosine 5′-phosphate in the presence of dicyclohexylcarbodiimide. These compounds have been obtained in relatively pure form in over-all yields ranging from 20 to 30 per cent by a combination of alcohol precipitation and treatment with Dowex 1 Cl^- 10 per cent cross-linked resin. The properties and analyses of L-methionyl adenylate indicate that the amino acid is linked to the phosphate group of adenosine 5′-phosphate in an acyl phosphate linkage.

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